Sperm capacitation is, after all, a prerequisite for both partial and complete acrosome reaction

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Abstract The acrosome reaction (AR) – an essential step in mammalian fertilization – can occur, according to the consensus, only in capacitated spermatozoa. In apparent contrast, recent reports have demonstrated that human spermatozoa incubated in vitro in an albumin-free medium and therefore believed to be non-capacitated, do undergo the AR. With the aim of determining unequivocally whether or not capacitation is required for the AR and whether albumin is essential for capacitation, we compared the potential to undergo partial and complete AR (induced by phorbol myristate ester or by the Ca^{2+} ionophore A23187) between human spermatozoa incubated in a capacitating medium, albumin-free medium, and non-capacitating medium. The results clearly demonstrate that capacitation is, after all, a prerequisite for both partial and complete AR. Albumin, on the other hand, is essential only for acquiring the capacity to undergo complete, not partial AR.

Key words: Capacitation; Acrosome reaction; Albumin; Human sperm; A23187; Phorbol ester

1. Introduction

For penetrating an egg and fertilizing it in vivo, mammalian spermatozoa must undergo the acrosome reaction (AR). This reaction involves fusion between the plasma membrane and the underlying outer acrosomal membrane, as a result of which the acrosomal contents, including a variety of hydrolytic enzymes which enable the penetration of the sperm to the egg, is released (see [1–3] for reviews). Spermatozoa are induced to undergo the AR in vivo when they bind to the egg's zona pellucida; in vitro they can also be induced by other inducers such as the Ca^{2+} ionophore A23187, progesterone, or phorbol myristate ester (PMA) [4].

There has been a consensus for years that not all the cells in a sperm population can undergo the AR upon induction; cells must be capacitated, i.e. they must be in a physiological state that confers on them the capacity to undergo this reaction ([3] for review). Capacitation occurs in vivo in the female genital tract [5], or in vitro as a result of seminal fluid removal followed by incubation in a capacitating medium (in humans for at least 40–130 min [6]). The composition of the in vitro capacitating medium varies from species to species, but in most species the capacitating medium contains proper ions (including Ca^{2+} and bicarbonate), energy substrates, and albumin [3]. There is apparently contradicting information in the literature with respect to the contribution of each of the medium components to capacitation. Thus, many reports ([3] for review) have indicated that there is no component whose presence in the medium is absolutely necessary, i.e. a component whose absence prevents capacitation. This has been demonstrated for K^+, Ca^{2+}, HCO_3^-, albumin and others [3]. On the other hand, a number of other studies have found [7,8] or assumed [9–12] that spermatozoa do not become capacitated unless albumin is present. Furthermore, since some of the latter studies [9–12] have demonstrated that spermatozoa pre-incubated in an albumin-free medium can be induced to undergo the AR in spite of the absence of albumin, they have concluded that, in vitro, capacitation is not absolutely essential for inducing the AR. The validity of this conclusion depends on whether or not albumin is required for capacitation. Our aim in this study was to try to resolve this conflict and to determine unequivocally whether or not capacitation is required for the AR and whether albumin is essential for capacitation.

2. Materials and methods

2.1. Chemicals

Anti-mouse immunoglobulin G (IgG), bovine serum albumin (BSA, fraction V powder), dimethyl sulfoxide (DMSO), A23187, PMA, pyruvic acid, propidium iodide, HEPES and FITC-PSA were obtained from Sigma Chemicals Company (USA). FITC-conjugated mouse anti-human CD46 and IgG were purchased from Serotec (UK). Percoll was purchased from Pharmacia (Sweden). All other chemicals were obtained from Merck (Germany). All the chemicals were of the highest purity available.

2.2. Media

The capacitating ('complete') medium used in this study was Biggers, Whitten and Whittingham (BWW) medium [13] (95 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM sodium lactate, 5 mM glucose, 0.25 mM sodium pyruvate and 25 mM $NaHCO_3$, pH 7.4) supplemented with HEPES (10 mM, pH 7.4) and albumin. Initially (in the experiments summarized in Table 1) we used human serum albumin (35 mg/ml final concentration) as in the study of Bielfeld et al. [12]. However, later we found experimentally that there is no difference between the results obtained with human serum albumin (at the mentioned excessive concentration) and BSA (at the routinely used concentration of 3 mg/ml) in the medium, and that 35 mg albumin/ml interfere with the function of A23187. Therefore, in the experiments shown in Fig. 1, we switched to work with BSA (3 mg/ml). The 'albumin-free medium' was essentially the complete medium, described above, but without albumin. The 'non-capacitating medium' (NCM) was the complete medium without albumin and $CaCl_2$, and with NaCl replacing $NaHCO_3$ (the total concentration of NaCl in this medium was therefore 120 mM).

2.3. Sperm preparation

Human semen samples were obtained from healthy donors having normal sperm counts, motility and morphology (according to WHO

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Abbreviations: AR, acrosome reaction; BWW, Biggers, Whitten and Whittingham medium; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; NCM, non-capacitating medium; PMA, phorbol myristate ester; PSA, *Pisum sativum* agglutinin

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Table 1
Effects of incubation time and albumin on the level of acrosome-reacted spermatozoa

Incubation period (min)	Presence of albumin	Acrosome-reacted spermatozoa (%)	
		Before PMA	After PMA[a]
0	+	5.3 ± 1.7 (3–7)	5.3 ± 1.3 (3–8)
60	+	5.0 ± 1.4 (3–6)	12.5 ± 0.6 (12–13)
0	−	5.5 ± 1.7 (3–7)	5.5 ± 1.3 (4–7)
60	−	6.0 ± 1.4 (4–7)	12.5 ± 1.9 (11–15)

The values shown are the mean ± S.D. (range) of 4 experiments, carried out with 4 sperm donors. The percentage of acrosome-reacted cells was calculated from the total number of spermatozoa (both motile and non-motile). When present, the concentration of human serum albumin in the incubation medium was 35 mg/ml. The incubation period was considered from the time of the preceding wash for seminal fluid removal. The small differences between the respective values in the absence and presence of albumin were statistically insignificant according to ANOVA-repeated measures. The difference between the AR level after PMA addition following 60 min incubation and the other AR levels was statistically significant at 99% according to the Fisher protected LSD test.
[a]Note that the incubation period in this column was essentially longer than mentioned, due to additional 30 min incubation at 35°C with the AR inducer PMA after the indicated time.

guidelines of 1992) after 3 days of sexual abstinence. For the experiments of Table 1, semen samples were divided into two portions. One portion was washed twice (20 min at 120×*g*) with the complete medium, and the other with the albumin-free medium. Each of the portions was divided into 3 sub-portions. In the first sub-portion, the percentage of acrosome-reacted spermatozoa – before and after induction with PMA – was determined immediately after the wash. In the second sub-portion, the spermatozoa were incubated at 35°C for 60 min and only then the AR level was determined (with and without PMA induction). In the third sub-portion, PMA was present throughout the incubation period (60 min at 35°C). For the experiments of Fig. 1, each semen sample was divided into 3 portions. The portions were separated on a discontinuous Percoll gradient (95% and 47.5% Percoll) [14] made in the complete medium, the albumin-free medium, or NCM, respectively. The gradient-containing tubes were spun in a centrifuge for 20 min at 300×*g*, and the 95% Percoll layer (containing mainly progressively motile spermatozoa) was recovered. The spermatozoa were washed twice with the respective medium (300×*g* for 10 min at room temperature) and resuspended in the same medium to a final sperm concentration of 2×10^7 cells/ml. Each sperm suspension was then re-divided into two sub-portions: one treated immediately with the AR inducer A23187, and the other treated with A23187 only after incubation for 3 h under an atmosphere of 5% CO_2 at 37°C.

2.4. AR induction

For AR induction with PMA, spermatozoa (10^8 cells/ml) were incubated with PMA (5 μM from a stock solution in DMSO; the final concentration of DMSO in the sperm samples did not exceed 0.5%) for 30 min at 35°C. For AR induction with A23187, spermatozoa (2×10^7 cells/ml) were incubated with A23187 (10 μM from a stock solution in DMSO; the final DMSO concentration was 0.2%) for 30 min at 37°C. In the case of NCM, $CaCl_2$ (1.3 mM) was added together with A23187 (experimental samples) or DMSO (control samples). The spermatozoa were then washed with PBS by centrifugation for 10 min at 300×*g* and resuspended in PBS. For AR determination, each sample was divided into two portions, one for detection by FITC-PSA and the other by FITC-CD46.

2.5. AR determination by FITC-PSA

Acrosome-reacted spermatozoa were identified by the acrosomal marker FITC-PSA essentially as described by Cross et al. [15], with the modifications introduced by Tesarik et al. [16], only that 100 μg/ml FITC-PSA for 10 min were used (instead of 50 μg/ml for 30 min) and that the slide was fixed with 2% formaldehyde after the staining. Each determination was carried out in duplicate. The slides were inspected blindly using Zeiss Axiovert 35 microscope equipped with a ×100 oil objective. As many as 200 cells were counted on each slide. Sperm motility was used as a marker for cell viability. Since the percentage of motile spermatozoa was similar before and after incubation with the AR inducers, there was no need to use supravital staining [4].

2.6. AR determination by FITC-CD46

Spermatozoa were first incubated for 30 min at room temperature with anti-mouse IgG (0.22 mg protein/ml) in order to block non-specific sites, followed by washing the blocker off with PBS (by centrifugation for 10 min at 300×*g*). The pellet was resuspended in PBS and divided into 2 aliquots. To one aliquot 1:50 diluted FITC-conjugated mouse anti-human CD46 was added, and to the other 1:50 diluted FITC-conjugated mouse anti-human IgG (a negative control). Following 30 min incubation at room temperature in the dark, the spermatozoa were washed with PBS and resuspended in 1 ml PBS containing 1% formaldehyde. Immediately prior to FACS analysis, the supravital probe propidium iodide [17] (2.5 μg/ml final concentration) was added. Samples of 10 000 cells (considered as 100%) were then analyzed by a Beckton Dickenson FACScan. The AR level was calculated for live cells (cells impermeable to propidium iodide) out of the total sperm population (both permeable and impermeable cells). The AR values shown are the differences between the signal obtained with FITC-CD46 and the control FITC-IgG.

2.7. Statistical analysis

Statistical analyses were performed with STATVIEW (BrainPower, Calabasas, CA) or InStat (GraphPad Software, San Diego, CA). The significance of the difference between the treatments was calculated by Student's *t*-test, ANOVA-repeated measures, and Fisher protected least significance difference (LSD) test, as appropriate.

3. Results

We compared, in two sets of experiments, between spermatozoa that are, by definition, capacitated and can be induced to undergo partial or complete AR[(1)], and spermatozoa that are, by definition, non-capacitated. In one set, capacitated spermatozoa were compared to spermatozoa that were assayed at time zero and therefore did not have a chance to become capacitated. In the other set, capacitated spermatozoa were compared to spermatozoa that were incubated in a medium that does not support capacitation. The inducers were PMA, which had been shown to induce partial AR only, and A23187, which induces both partial and complete AR (B. Jaiswal, M. Eisenbach and I. Tur-Kaspa, submitted).

3.1. Comparison according to time

Two aliquots of the same sperm samples, one containing and one missing albumin, were each divided to two sub-aliquots. One sub-aliquot was examined immediately after washing off the seminal fluid ($t=0$; defined as non-capacitated spermatozoa because of having no time for capacitation),

(1) The terms partial and complete AR refer to the acrosomal status of individual spermatozoa rather than to the relative number of acrosome-reacted cells. By partial AR we mean partial release of the acrosomal content and/or loss of the outer acrosomal membrane. By complete AR we mean complete exposure of the inner acrosomal membrane and complete loss of the acrosomal content [18].

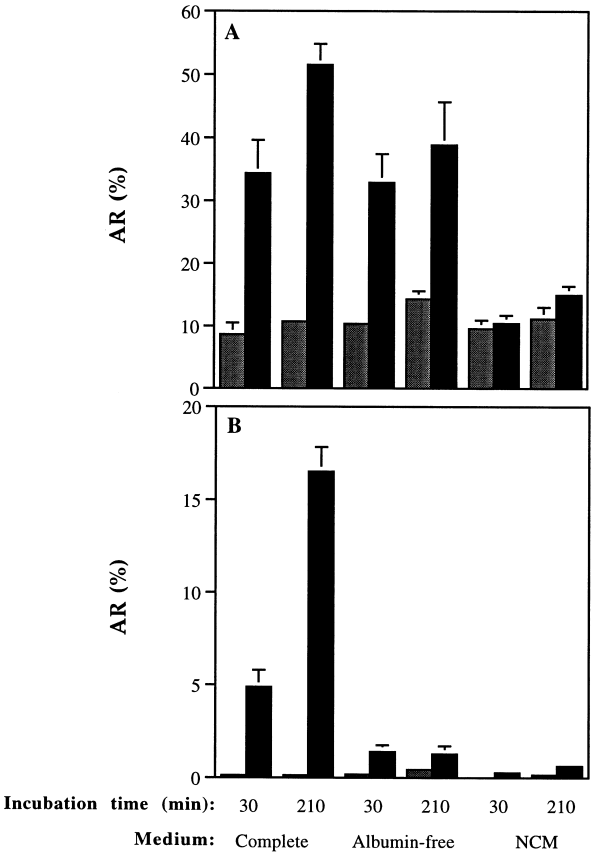

Fig. 1. Spontaneous and induced AR levels in complete, albumin-free, and NCM media. Note that the mentioned incubation time includes the incubation period in one of the three media (0 or 180 min) plus 30 min incubation with the AR inducer A23187 (black columns) or with the no-inducer control (gray columns). The no-inducer control samples (spontaneous AR) contained DMSO (0.2% final concentration), as did the experimental samples. The values shown are the mean ± S.E.M. of four experiments with four different sperm donors. It should be noted that error bars were added to all the columns; in those columns where they appear to be absent, they are too small to be distinguished. A: AR measurement with FITC-PSA. B: AR measurement with FITC-CD46. Note that the *y*-axis scales are different in panels A and B. In panel A, the statistical significance of the difference between spontaneous and A23187-induced AR, calculated by a paired Student's *t*-test, was $P < 0.02$ at 30 min and $P < 0.002$ at 210 min for the complete medium, and $P < 0.02$ and $P < 0.04$ for the albumin-free medium. The difference for NCM was insignificant at 30 min but significant at 210 min ($P < 0.05$). In panel B, the statistical significance of the differences between spontaneous and induced AR were $P < 0.02$ at 30 min and $P < 0.002$ at 210 min for the complete medium. The differences in the other two media were insignificant.

and the other was assayed after additional 60 min incubation in the complete (capacitating) medium. As shown in Table 1, PMA induced spermatozoa to undergo the AR (beyond the spontaneous level of AR) only after the 60 min incubation period. This observation confirmed that there were no capacitated spermatozoa at $t = 0$, and demonstrated that some spermatozoa ($\sim$7.5% of the total population) became capacitated during the incubation period. (The difference between the levels of acrosome-reacted cells before and after PMA induction was considered as the level of capacitated spermatozoa.) Similar results were obtained in the absence of albumin (Table 1). The level of acrosome-reacted cells was independent of the incubation time with PMA. When other aliquots of the same sperm samples were incubated with PMA from the very beginning ($t = 0$ to $t = 60$ min), the results were not different: the mean level of acrosome-reacted cells was 11.3 ± 1.7% both in the presence ($n = 4$) and absence ($n = 4$) of albumin (range 9–13%). The above-mentioned observations indicate that sperm incubation under capacitating conditions is a prerequisite for AR induction. They further suggest that, under these experimental conditions, albumin is not essential for AR induction by PMA as detected by FITC-PSA.

3.2. Comparison according to medium composition

It has been shown that removal of Ca^{2+}, HCO_3^- or albumin from the sperm suspending medium prevents [8] or reduces [3] capacitation. Therefore, as a second means to have spermatozoa that are, by definition, non-capacitated, we used cells suspended in a medium missing these three components together (termed NCM). We compared the AR levels of spermatozoa in three media: complete, albumin-free, and NCM. Since PMA – the AR inducer used in the first set of experiments-can only induce partial AR[1] (Jaiswal et al., loc. cit.), we extended the study to conditions that can induce complete AR. We used the Ca^{2+} ionophore A23187 (in the presence of Ca^{2+}) which is the only inducer – of those commonly used in vitro – that induces a complete AR within 3 h of incubation (Jaiswal et al., loc. cit.). As shown in Fig. 1A which includes the results obtained with FITC-PSA, the level of spontaneous AR (gray columns) was not significantly different between the media tested. However, after induction with A23187, the level of acrosome-reacted cells increased in the complete and albumin-free media but not in NCM (black columns). This observation confirmed that there were no capacitated spermatozoa in NCM and demonstrated that a similar percentage of spermatozoa acquired the capacity to undergo induced AR in the complete and albumin-free media. Thus, here too, as in Table 1, albumin did not appear to be essential for capacitation as measured by FITC-PSA from the difference between induced and spontaneous AR. Interestingly, a 30-min incubation period appeared to be almost sufficient for acquiring the capacity to undergo A23187-stimulated AR (compare the 30- and 210-min columns in Fig. 1A).

FITC-PSA is a probe that recognizes both partial and complete AR ([18] and Jaiswal et al., loc. cit.). To distinguish between the two, we used FITC-CD46 which – as a positive staining probe – identifies complete AR only [18–21]. With this probe, the picture was somewhat different. As shown in Fig. 1B, the level of spontaneous AR was close to zero in all the media tested, in agreement with our earlier observations that the spontaneous AR is partial and therefore cannot be detected by CD46 (Jaiswal et al., loc. cit.). Similarly to Fig. 1A, A23187 increased the level of acrosome-reacted spermatozoa in the complete medium but not in NCM, endorsing the conclusion that capacitation is a must for the induced AR. In contrast to Fig. 1A, however, here the difference between the AR levels after 30- and 210-min incubation periods in the complete medium was significant ($P < 0.03$). Also, in the absence of albumin, the A23187-induced AR which could be detected by FITC-PSA (Fig. 1A), was hardly detected by FITC-CD46 (Fig. 1B). These observations suggest that albumin is not essential for acquiring the capacity to undergo partial AR, but it is essential for acquiring the capacity to undergo complete AR. Similarly, they suggest that a 30-min

incubation period is sufficient for capacitating cells to undergo partial but not complete AR[(2)].

4. Discussion

The following main conclusions may be drawn from this study. (i) Spermatozoa can be induced to undergo the AR, both partial and complete, only after capacitation. (ii) Albumin is not essential for gaining the capacity to undergo partial AR; it is, however, required for complete AR. These conclusions are discussed in depth below.

4.1. Requirement of capacitation for the AR

Two lines of evidence indicate that capacitation is a prerequisite for the AR: spermatozoa assayed at time zero (before having a chance to become capacitated; Table 1) and spermatozoa incubated in a medium that does not support capacitation (because of the absence of albumin, Ca^{2+} and HCO_3^- that, together, are essential for capacitation; Fig. 1) could not be induced to undergo the AR. In contrast, spermatozoa of the same samples did undergo the AR if allowed to incubate in a capacitating medium. These observations thus confirm unequivocally that capacitation is a must for induced AR. Furthermore, the study indicates that both partial and complete AR require capacitated spermatozoa.

An interesting observation is that a period of 30 min incubation with A23187 was sufficient to induce the AR (Fig. 1), whereas a 30-min incubation with PMA (shown in Table 1 as time zero) was not sufficient. The difference is probably that A23187 induces Ca^{2+} influx instantaneously, whereas PMA works by stimulating physiological processes (possibly by activating Ca^{2+} channels in the sperm plasma membrane [22]) which require time. Thus it seems that spermatozoa which became capacitated at close to 30 min had sufficient time to undergo the AR by A23187 but not by PMA.

4.2. Requirement of albumin for capacitation and AR

The question of whether or not albumin is required for sperm capacitation in humans, apparently cannot be simply answered by 'yes' or 'no'. Our observations suggest that albumin is not essential for partial AR, induced by PMA (Table 1) or A23187 (Fig. 1A), as measured by the negative-staining probe FITC-PSA. However, it appears to be essential for complete AR, as measured in a cell sorter by the positive-staining probe FITC-CD46 (Fig. 1B). Two potential explanations may be given to these selective requirements. (i) Capacitation is a multi-step process ([23,24] for reviews). It is possible that albumin is not required for the steps which make a spermatozoon partially capacitated but it is required for the subsequent steps that make it fully capacitated. According to this possibility, a partially capacitated cell cannot be induced to undergo a complete AR; it can only undergo a partial AR. Fully capacitated cells, on the other hand, can undergo a complete AR. (ii) The albumin requirement might be for complete AR, not for complete capacitation. This possibility is in accordance with an earlier suggestion of Fraser [25], but it is not in accordance with our recent observations that the AR level (both partial and complete) in spermatozoa incubated in the presence of albumin for full capacitation and then with A23187 for AR induction, appears to be unaffected by whether or not albumin is present during the AR induction step (B. Jaiswal and M. Eisenbach, unpublished). We therefore favor the first possibility. Taken together, our results suggest that albumin is essential for the processes involved in changing partially capacitated spermatozoa to fully capacitated, but it is not a essential for partial capacitation and for the AR.

It was suggested that albumin participates in cholesterol removal from the plasma membrane, which is one of the processes involved in capacitation [26–32]. If so and if indeed albumin is required for changing partially capacitated spermatozoa to fully capacitated ones, then cholesterol removal appears to be one of the later steps in capacitation.

4.3. Correlation with earlier observations

The results of this study are in agreement with a large number of studies indicating that capacitation is a prerequisite for induced AR ([3,24] for reviews). Capacitation was demonstrated, even in a cell-free system, to be a prerequisite for membrane fusion during the AR [33]. However, on the basis of observations that human spermatozoa, pre-incubated in an albumin-free medium and considered non-capacitated, can be induced to undergo the AR, a number of studies have concluded that, at least in vitro, capacitation is not a prerequisite for induced AR [10–12]. The observations made herein resolve the apparent conflict. Although in those studies the spermatozoa were not incubated under classical capacitating conditions, they were incubated with an AR inducer. For example, Bielfeld et al. [12] incubated spermatozoa with A23187, PMA or other AR inducers for 60 min in an albumin-free medium. They identified acrosome-reacted spermatozoa by the double-stain technique which is a negative-staining technique that can identify also partially acrosome-reacted spermatozoa. As in Table 1 (which summarizes experiments carried out on purpose under conditions similar to those of Bielfeld et al.) and Fig. 1A, Bielfeld et al. found acrosome-reacted spermatozoa in spite of the absence of albumin. Even though their results are consistent with ours, they apparently reached a wrong conclusion that capacitation may not be essential for induced AR because they presumed that spermatozoa in the absence of albumin are non-capacitated. In other words, Bielfeld et al. who apparently meant to compare between the ability of capacitated and non-capacitated spermatozoa to undergo the AR, may have dealt with two populations, both of which were apparently capacitated: one fully and one partially.

It has been shown that, in the presence of albumin, the ability of hamster spermatozoa to penetrate the female egg is ~40% larger than in the absence of albumin [34], and the ability of mouse spermatozoa to fertilize the egg is 40–60% larger [25]. Furthermore, in both species, the fertilized eggs reach more advanced stages of nuclear development in the presence of albumin [25,34] and, in hamsters in the absence of albumin, most of the penetrated spermatozoa remain in the perivitelline space [34]. Since the ability of spermatozoa to penetrate and fertilize an egg is one of the best criteria for

[(2)] Note that Fig. 1A shows the total level of acrosome-reacted spermatozoa (i.e. the sum of the cells that underwent complete AR and the cells that underwent partial AR), whereas Fig. 1B shows the level of cells that underwent complete AR only. Therefore, when the AR is mainly partial and the level of cells with complete AR is very low (e.g. after 30 min incubation), the AR level is high in panel A and low in panel B. Following 210 min incubation, a significant portion of the cells with partial AR become completely acrosome-reacted. For this reason, the main increase in the AR level at 210 min is in panel B.

being fully capacitated and undergoing the AR, these observations are in accordance with the notion that albumin is required for full capacitation.

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